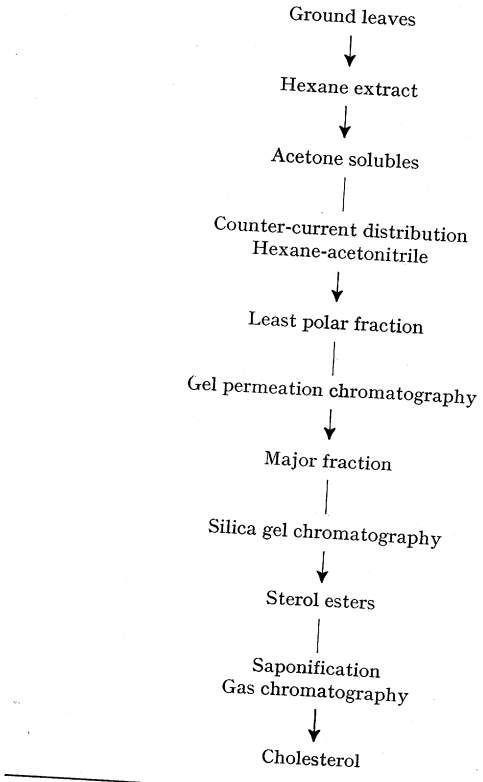
Cholesteryl Esters in Flue-Cured Tobacco

The constituents of tobacco in all its various forms have received extensive chemical study[1], and several sterols, sterol esters, and sterol glycosides have been reported in tobacco – the sterols commonly obtained being stigmasterol, β-sitosterol, ergosterol and campesterol (JOHNSTONE et al.[1,2]). Cholesterol, often considered to be an 'animal' sterol, has been found only recently in plants and to our knowledge has been conclusively identified in only 4 species[3] although its presence has been inferred in others[4]. In spite of extensive work on tobacco, cholesterol and its derivatives have never been isolated from this plant[5]. We now report a simple sequence of separation steps (chart) which results in the ready isolation of a sterol ester fraction from the hexane extract of flue-cured tobacco, together with proof of the presence of a significant percentage of cholesteryl esters in this fraction.

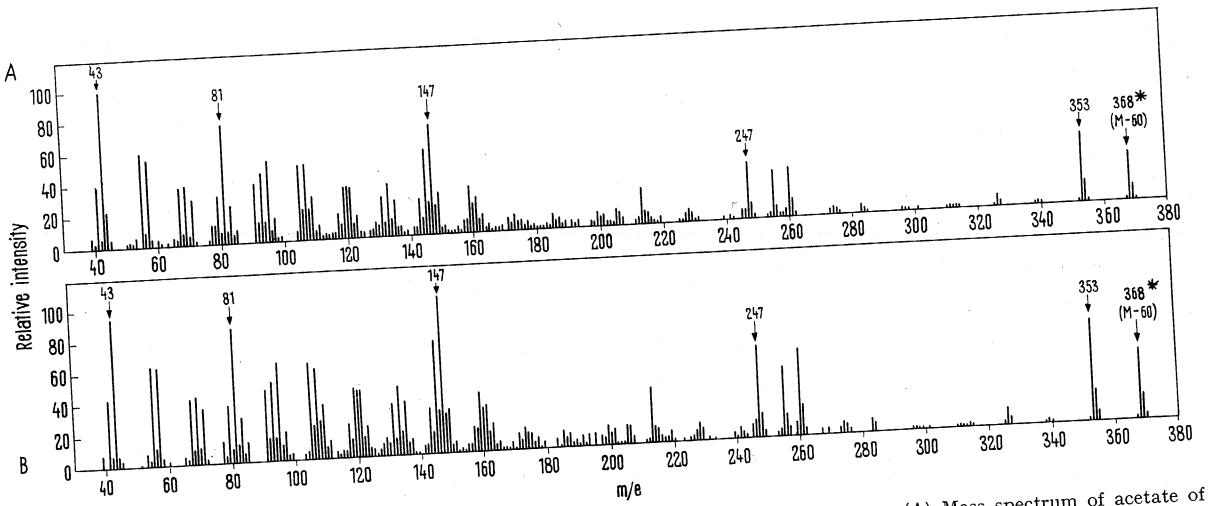
Flue-cured tobacco leaves[6] were ground and extracted continuously with warm hexane[7]. The extract was chilled, filtered, and evaporated, and the residue (5%[8]) was dissolved in acetone and chilled. Precipitated solids were removed by filtration and the acetone evaporated. The residue (4.5%) was subjected to a 14 transfer countercurrent distribution using hexane as the stationary phase and acetonitrile as the mobile phase. The residue from evaporation of tube 0 (i.e., the least polar material, 1.3%) was chromatographed on a column of 200–400 mesh polymer beads (polystyrene cross-linked with 2% divinylbenzene[9]) swollen with benzene. Elution was carried out with benzene. Several fractions were obtained, roughly in order of decreasing average molecular weight, as would be expected for this type of chromatography.

The major fraction (0.54%), indicated by IR-spectra to be a mixture of hydrocarbons, esters and alcohols, was dissolved in hexane and adsorbed onto a silica gel column (Davison silica gel, 100–200 mesh, 100:1 weight ratio). Elution with hexane removed hydrocarbons. Most of the other material was removed using gradient elution from pure hexane to pure ether. Two main bands were obtained.

Chart



The more polar of these (0.25 %) was shown to be solanesol by its melting point and the melting point and mixed melting point of its *p*-phenylazobenzoyl ester. IR-spectra indicated the less polar fraction (0.11%) to be composed of esters. Separation of this fraction and identification of individual esters was not carried out. Instead the total material was saponified to give an acid fraction (indicated by gas chromatography of the methyl esters to be a mixture of C_{14}-C_{18} fatty acids with palmitic, stearic and oleic acids predominating) and an alcohol fraction. The latter was inseparable from cholesterol and stigmasterol by thin layer chromatography on silica gel in 10% acetone-



Comparison of mass spectrum of genuine cholesterol with that of sterol isolated from tobacco. (A) Mass spectrum of acetate of GC peak 1, 100° direct probe, 2.0 Kv dynode. (B) Mass spectrum of cholesteryl acetate, 150° direct probe, 2.5 Kv dynode. * Reduced by factor of 10.

hexane. Gas chromatography of the alcohols as the dimethylsilyl ethers (column of 1% OV-17 on acid washed, silanized Chromosorb W) showed 4 principal components with relative retention times of 0.74, 1.00, 1.09 and 1.26. These are quite close to the relative retention times reported for the trimethylsilyl ethers of cholesterol, campesterol, stigmasterol and β-sitosterol (0.78:1.00:1.10:1.25)[10]. Admixture of genuine cholesterol and stigmasterol followed by silylation enhanced the peaks assigned to these compounds.

The 4 major components were isolated by means of preparative gas chromatography of the dimethylsilyl ethers. The collected substances (each of which gave only 1 peak on gas chromatograms) were boiled with aqueous methanol for removal of the silyl groups and the free alcohols were acetylated. Mass spectra were then obtained. Comparison of the mass spectrum of the acetate from the first peak with that of genuine cholesteryl acetate (Figure) left no doubt as to its identity. The isolation of stigmasteryl, campesteryl and β-sitosteryl derivatives is unexceptional, and their identification is based on the gas chromatographic retention times, supported by mass spectra of the isolated acetates which had M-60 peaks at M/e 394, 382 and 396, respectively. (In contrast to the well-separated cholesterol, there was some cross-contamination among these 3 sterols.)

The combination of counter-current distribution and gel permeation chromatography[11] provides a convenient and exceptionally mild method for dividing a complex extract into groups of materials which are then amenable to handling by more usual techniques. In this case the sterol esters were separated rather cleanly from other ester material and were contaminated only by materials such as solanesol which were easily separable by conventional adsorption chromatography. Analytical gas chromatography showed that cholesteryl esters represented ca. 5% of the phytosteryl ester mixture. Thus these esters represent about 0.005% of the weight of the tobacco[12].

Zusammenfassung. Nach Hydrolyse wurde Cholesterol von einem Sterolester-Bruchteil aus dem «flue-cured» Tabak isoliert und massenspektrometrisch sein Acetat-Derivat einwandfrei festgestellt, obwohl die einzelnen Ester von Cholesterol im Tabak nicht gereinigt oder identifiziert wurden. Zur Isolierung wurden die Zwei-Stufen-Gegenstromverteilung und die Gel-Permeations-chromatographie verwendet, 2 brauchbare und wenig aggressive Methoden zur Aufteilung eines komplexen Extraktes in Materialgruppen und nachfolgender gewöhnlicher chromatographischer Bearbeitung.

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[1] R. A. W. JOHNSTONE and J. R. PLIMMER, Chem. Rev. *59*, 885 (1959); R. L. STEDMAN, A. P. SWAIN and W. RUSANIWSKYJ, Tob. Sci. *6*, 1 (1962); A. P. SWAIN, W. RUSANIWSKYJ and R. L. STEDMAN, Chem. Ind. 435 (1961).

[2] 'γ-Sitosterol has been shown to be a mixture of β-sitosterol and campesterol. M. J. THOMPSON, W. E. ROBBINS and G. L. BAKER, Steroids *2*, 505 (1963); I. NISHIOKA, N. IKEKAWA, A. YAGI, T. KAWASAKI and T. TSUKAMOTO, Chem. pharm. Bull., Tokyo *13*, 379 (1965).

[3] D. F. JOHNSON, R. D. BENNETT and E. HEFTMANN, Science *140*, 198 (1963); R. D. BENNETT, S. T. Ko and E. HEFTMANN, Phytochem. *5*, 231 (1966); B. A. KNIGHTS and W. LAURIE, Phytochem. *6*, 407 (1967); M. DEVYS and M. BARBIER, C. r. hebd. Séanc. Acad. Sci., Paris *261*, 4901 (1965).

[4] See, inter alia, M. F. HÜGEL, W. VETTER, H. ANDIER, M. BARBIER and E. LEDERER, Phytochem. *3*, 7 (1964); P. DUPERON, W. VETTER, M. BARBIER, Phytochem. *3*, 89 (1964); C. DJERASSI, J. C. KNIGHT and H. BROCKMANN JR., Chem. Ber. *97*, 3118 (1964); J. W. ROWE, Phytochem. *4*, 1 (1965).

[5] P. BENVENISTE, L. HIRTH and G. OURISSON reported that the sterol fraction from tobacco tissues grown in vitro contained a minor constituent (1% or less), the molecular weight of which corresponded to that of cholesterol, but conclusive identification was not made. Phytochem. *5*, 31 (1966).

[6] The tobacco was Hicks variety, government grade B4 LV, flue-cured tobacco, harvested and purchased in 1964, and stored in a freezer.

[7] M. Dymicky and R. L. STEDMAN, Tob. Sci. *3*, 179 (1959).

[8] Percentages are approximate and are given in terms of the undried leaf.

[9] Generously provided by the Dow Chemical Company.

[10] A. ROZANSKI, Anal. Chem. *38*, 36 (1966).

[11] Gel permeation chromatography on polystyrene-divinyl benzene polymers has been applied chiefly to polymer mixtures [J. C. Moore, J. Poly. Sci. *2A*, 835 (1964)]. An example of its application to lipids has been recorded by C. L. TIPTON, J. W. PAULIS and M. D. PIERSON, J. Chromat. *14*, 486 (1964). We thank Dr. TIPTON for helpful correspondence.

[12] A report of work done under contract with the U.S. Department of Agriculture and authorized by the Research and Marketing Act of 1946. The contract is being supervised by the Eastern Utilization Research and Development Division of the Agricultural Research Service. Mass spectra were obtained by Dr. MAURICE BURSEY of the University of North Carolina.